

## THE AMINO ACID COMPOSITION OF MAJOR AMPULLATE GLAND SILK (DRAGLINE) OF *NEPHILA CLAVIPES* (ARANEAE, TETRAGNATHIDAE)

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### ABSTRACT

Amino acid composition of major ampullate gland silk (dragline) produced by the mature, female golden orb-weaving spider, *Nephila clavipes* was determined. Several solvents were applied in order to solubilize the spider silk. Although several strong acids and bases were able to solubilize silk, the protein was apparently degraded by this treatment, as demonstrated by protein gel electrophoresis. Only a mixture of hydrochloric/propionic acid (50:50, v.v, final concentration 3N HCL/25% propionic acid) solubilized the silk while retaining the molecular weight integrity of the crystalline polymer. The results show that the major ampullate gland secretion is characterized by a high degree of small side chain amino acids (Ala, Gly, and Ser) and polar residues (Gly and Arg), comprising almost 75% of the total amino acids present. Contrary to published findings (Work and Young 1987), the composition of major ampullate gland silk appears to be uniform within the species. The composition of the secretion is discussed in relation to the known and implied functions of the major ampullate gland as well as in relation to the mechanical properties of the silk produced by orb-web building spiders.

### INTRODUCTION

Spiders are unique in their ability to synthesize and utilize silks for a variety of purposes. The orb-web spinners are equipped with 5-7 different types of silk secreting glands, each synthesizing its own type of silk to be utilized for a specific purpose, e.g., construction of the dry and sticky parts of the web, construction of the egg-sac, and swathing silk of captured prey (Gosline et al. 1984). These fibers are synthesized by extremely specialized glands situated in the abdominal cavity. Although the amino acid composition is known for the seven silks from one animal (Andersen 1970), only two of the seven types of silk have been investigated in any detail. *Nephila clavipes* is a large, orb-weaving spider, dispersed in the tropical and subtropical areas of the western hemisphere (Moore 1977). Their most prominent glands are a pair of large major ampullate glands which secrete the protein for dragline silk. Three morphological regions distinguish the gland: the tail, ampulla, and duct. The tail is the site of approximately 90% of the major ampullate gland's protein synthetic activity; the ampulla is a storage site for soluble dragline silk; and the duct appears to be involved with secretion and ordering of silk (Bell and Peakall 1969). It can be assumed that the mechanoelastic properties of the silk fibers correlate closely

with their functional properties and that these properties are in turn determined by their chemical composition and molecular conformation. The multiformity of material makes spider silk ideal for studies on the relationship between chemical composition, structural conformation, and mechanoelastic properties of biological fibers.

The term fibroin is often used for the silk fibers secreted by some insects and arachnids (Lucas et al. 1958). Studies on the chemistry of insect and arachnid fibroins have been previously reported by Rudall (1962), Lucas et al., (1960), Andersen (1970), Hunt (1970), Hazan et al., (1975), Tillinghast and Christenson (1984), and Work and Emerson (1987). Data on *Nephila* silk amino acid composition is limited. Amino acid composition has been reported to a lesser degree for *Nephila senegalensis* (Walkenaer) (Lucas et al. 1960), *Nephila madagascariensis* (Vinson) (Lucas et al. 1960), and *N. clavipes* (Zemlin 1967; Tillinghast and Christenson 1984). The silks of these organisms appear to be composed of anti-parallel beta-pleated sheets but have different intersheet distances (Warwicker 1960). These investigations imply that the silks vary in composition and properties, but there is insufficient information to make a definitive correlation between chemical composition and structural properties. X-ray diffraction patterns (Gosline et al. 1984, 1986) have implied that the molecular conformation of major ampullate gland fibers consists of crystalline regions dispersed in a matrix of amorphous proteinaceous material. The ratios of crystalline to amorphous regions may be a crucial factor in the assessment of physical properties of the fiber.

The objectives were to (1) develop a system by which silk fibers obtained by controlled silking could be completely solubilized while retaining the molecular weight integrity of the fiber, (2) determine the amino acid composition in major ampullate gland silk (MaAS) of *N. clavipes*, and (3) search for correlations between MaAS chemical composition and physical properties of these fibers. In this paper we describe the results of amino acid composition analysis of the dragline silk of *N. clavipes* and bring out the importance of the relationships between chemical composition and physical properties.

## MATERIALS AND METHODS

**Species.**—Samples were collected from the following araneid species, *N. clavipes* Nephilinae were kindly supplied by Angela Choate, University of Florida, Gainesville, FLA; *Argiope aurantia* (Lucas) and *Neoscona domiciliorum* (Hentz) were supplied by Mark Stowe, University of Florida, Gainesville, FLA. Specimens were kept alive in individual cages and fed a diet of German cockroaches, *Blattella germanica* (Blattellidae).

**Silk collection.**—Controlled silking was performed as described by Work and Emerson (1982). Controlled silking was restricted to the spiders which were large enough to be easily manipulated without damaging the spider. The silking procedure averaged 30 minutes and 5.0 milligrams (mg) of MaAS was routinely obtained. The mature females were continuously observed under 60X magnification to substantiate the glandular source of silk. All reeled samples were examined using a Zeiss light microscope (1250X total magnification) to ensure that there was no contamination by minor ampullate gland fibers.

**Silk solubilization.**—Silk samples (1.0-2.0 mg) were placed in 13 × 100 mm sterile glass borosilicate test tubes. The solvents listed in Table 1 were added to a final concentration of 1.0 ug/ul and solubility determined visually at room temperature.

**Removal of solvent.**—After solubilization the samples (reeled or glandular) were either dialyzed against 100 ml of 10 mM Tris-HCl, pH 7.0 for 24 h or dried immediately under vacuum (purged with argon) and reconstituted in the Tris buffer (final concentration 1 ug/ul).

**Silk hydrolysis.**—Major ampullate gland silk (reeled samples, 2.0 mg) were first dissolved in 2.0 ml of a hydrochloric/propionic acid mixture at room temperature for 20 min with slight vortexing. Solubilized samples (100 ul at 1.0 ug/ul) were vacuum dried in pyrolyzed vials and purged with argon gas. Hydrolysis was carried out by placing 200 ul of constant boiling 6N HCl in the bottom of the reacti-vial along with two sodium sulfite crystals. The vessel was again purged with argon gas, sealed under vacuum and placed at 150 °C for 1 hour. Argon was used as a purging gas because of its purity and because it contributes fewer artifact peaks in the subsequent analysis. Sodium sulfite is used as an oxygen scavenger and aids in the recovery of cysteine, serine, and threonine. The oxygen scavenging activity of the crystals in the reaction aids in avoiding non-specific hydrolysis of amino acid residues and subsequent amino acid degradation at the elevated temperatures (Ted Tanhauser personal communication).

**Amino acid analysis.**—Multiple analyses were carried out on a Waters HPLC Pico-Tag Amino Acid analysis system. The hydrolyzed samples were derivatized with phenylisothiocyanate (PITC) and these samples reconstituted in 400 ul of sample diluent. For each analysis a 50 ul injection volume was used. Amino acid standards were run with each sample. Ribonuclease A was run as an hydrolysis control.

**Glandular dissection.**—Major ampullate glands (tail, ampulla, and duct) were dissected out of living spiders through a 1.5 cm longitudinal incision along the ventral abdomen. The glands were removed carefully to avoid degradation of the luminal contents. The glands were immediately transferred to a medium containing 0.10M sodium chloride and 0.015 M sodium citrate (SSC). Protease inhibitors, phenylmethyl sulfonyl flouride (PMSF) at a final concentration of 6-10 mg/ml (Weber et al. 1972) and 20 units/ml of aprotonin (Piperno et al. 1979), were added to the dissection buffer to inhibit proteases released by the gastric system of the spider. Solubilization, hydrolysis, and amino acid analysis were performed as previously described.

## RESULTS

**Silk solubility.**—Of the solubilizing agents studied, only hydrochloric/propionic acid (50:50, v:v) dissolved *N. clavipes* dragline silk at room temperature with only slight agitation (Table 1). Hydrochloric acid below 6N and used alone failed to completely dissolve the silk even at elevated temperatures (data not shown). Some quarternary ammonium compounds used as commercial tissue solubilizers proved to be efficient solvents, but the solvent could not be easily removed from the solution. High concentrations of base also dissolved silk samples, although they were not used because the elevated temperatures needed for solubilization may

Table 1.—Solubility of *Nephila clavipes* dragline silk in various solvent systems. 1 = Totally insoluble, 2 = Partially soluble, some particulates, 3 = Partially soluble, no particulates, viscous suspension, 4 = Totally soluble, no particulates, clear, non-viscous.

Solvent	Solubility at room temperature
Water	-1
1N HCl	-1
2N HCl	-1
3N HCl	-1
4N HCl	-2
5N HCl	-2
6N HCl	-/+2
1N KOH	-1
Chloroform	-1
Ethyl alcohol 95%	-1
8M Urea	-2
50% Lithium Bromide	-2
1% SDS	-1
5% Mercaptoethanol	-1
Solvent	+3
Constant boiling 6N HCl/50% Propionic acid	+4

begin random hydrolysis of the silk backbone prior to amino acid hydrolysis. Any amino acids hydrolyzed prior to the 150 °C hydrolysis reaction may then become completely degraded at the hydrolysis step and subsequently unaccounted for in the final analysis (Ted Tanhauser personal communication).

Hydrochloric/propionic acid proved to be most suitable; it solubilized the silk immediately and more importantly retained the molecular weight integrity of the silk as determined by polyacrylamide gel electrophoresis and high performance liquid chromatography (data not shown).

**Amino acid analysis.**—The amino acid composition of the secretion of (MaAS) from *N. clavipes* is shown in Tables 2 and 3. Glycine, alanine, glutamic acid/ glutamine, and arginine were the most abundant amino acids, together comprising 74 percent of the total amino acids present. Generally, the major ampullate gland silk has been considered for use in the production of dragline, frame threads, and radii of the web. The dragline has a high tensile strength (198 grams per denier, gpd) and it has a rupture elongation of 18% (Zemlin 1967). The composition of the material from the large ampullate gland (pulled and glandular) generally agrees with the published analyses of dragline from *N. clavipes* (Zemlin 1967; Work and Young 1987), but some differences are observed. Work and Young 1987, report extremely low levels of asparagine, threonine, arginine and valine (0.87, 0.31, 1.37, and 0.76 respectively). We report significantly higher levels of these residues (see Table 2), theorizing that these residues play important roles in the amorphous domains of the polymer. Deoxyribonucleic acid (DNA) sequencing of the MaAS gene has confirmed the presence of these residues.

Table 3 shows the amounts of various amino acid side chains in dragline silk of *N. clavipes*. Dragline silk is composed predominantly of the small side-chain amino acids glycine, alanine, and serine, which would allow them to conform to the antiparallel beta-pleated sheet model proposed by Pauling and Corey (1953) for *Bombyx mori*. The conformational model applies only to the crystalline

Table 2.—Amino acid composition of reeled dragline silk of *Nephila clavipes*. Results expressed as residues per 100 total. Three trials each spider.

Amino acid	Spider 1	Spider 2	Spider 3
Asp/Asn (D/N)	2.5	2.4	2.6
Glu/Gln (E/Q)	9.1	9.0	9.2
Ser (S)	4.5	4.5	4.4
Gly (G)	37.0	37.3	36.9
His (H)	0.5	0.4	0.4
Arg (R)	7.6	7.6	7.7
Thr (T)	1.6	1.7	1.6
Ala (A)	21.1	21.0	21.2
Pro (P)	4.3	4.3	4.3
Tyr (Y)	3.0	3.0	3.2
Val (V)	1.8	1.8	1.7
Met (M)	0.3	0.3	0.2
Cys (C)	0.1	0.1	< 0.1
Ile (I)	1.0	1.0	1.0
Leu (L)	3.8	3.7	3.7
Phe (F)	0.7	0.7	0.7
Lys (K)	1.0	1.0	1.0

regions of *B. mori*, which makes up approximately 40% of the total silk structure, as described by x-ray diffraction analysis (Iizuka 1965). Limited x-ray diffraction data has been reported which describes the degree of crystallinity in dragline silk of certain araneid species, (Gosline et al. 1984, 1986, 1988).

We thought it worthwhile to look at the pulled draglines from other spider species, *Argiope aurantia* and *Neoscona domiciliorum*, and look for comparisons/differences in the amino acid compositions. Reeled samples of dragline silk were prepared as previously described. Table 4 shows the differences in the amino acid composition of the various draglines as compared to *Nephila clavipes* reeled dragline. Generally, *Argiope* and *Nephila* dragline silks are quite similar, although *Nephila* contains many more arginine residues (7.6% vs 2.9%). The arginine residue appears to be an important component of the amorphous domain repeating segment, as seen in DNA sequencing of the dragline silk gene (unpublished data). *Neoscona* dragline also has a similar amino acid composition

Table 3.—Amounts of various amino acid side chains in reeled dragline silk of *Nephila clavipes*. Results expressed as residues per 100 total. Small side chains: gly + ala + ser, polar residues: asp + glx, basic side chains: lys + his + arg cyclic imino side chain: pro, aromatic side chain: phe + tyr, sulfur containing: met + cys, aliphatic side chain: ala + val + ile, hydroxyl side chain: ser + thr. Three trials each spider.

Dragline silk	Spider 1	Spider 2	Spider 3
Small side chains	62.28	62.92	62.59
Polar side chains	29.81	29.61	30.22
Acidic/amide side chains	11.67	11.52	11.83
Basic side chains	9.05	9.02	9.06
Cyclic imino side chain	4.3	4.34	4.28
Aromatic side chain	3.62	3.57	3.88
Sulfur containing	0.47	0.46	0.22
Aliphatic side chain	27.61	27.57	26.62
Hydroxyl side chain	6.16	6.20	6.09

Table 4.—Amino acid composition of the silk gland secretions of various spiders. Results expressed as residues per 100 total residues.

Amino acid	<i>Nephila clavipes</i>		<i>Argiope aurantia</i>	<i>Neoscona domiciliorum</i>
	Dragline (reeled)	Glandular (MaAs)	Dragline (reeled)	Dragline (reeled)
Asx	2.5	2.1	1.6	0.6
Glx	9.2	8.3	11.1	10.0
Ser	4.5	3.9	5.1	6.8
Gly	37.1	38.1	34.7	38.0
Arg	7.6	7.2	2.9	0.6
Thr	1.7	2.0	0.8	0.9
Ala	21.1	23.4	22.2	18.0
Pro	4.3	3.9	6.4	11.2
Tyr	2.9	4.3	3.8	3.7
Val	1.8	1.7	1.5	0.7
Met	0.4	0.4	0.3	0.2
Cys	0.1	0.9	0.3	0.7
Ile	0.9	0.5	0.8	0.5
Leu	3.8	4.0	4.2	1.2
Lys	0.5	1.0	0.5	0.2

profile to *Nephila*, but does contain almost three times as many proline residues (4.3% vs 11.2%).

Table 4 also compares the amino acid composition between reeled and glandular sources of *Nephila clavipes* dragline silk. The data clearly shows the profiles are virtually identical in composition. Samples were prepared for analysis as described in materials and methods.

## DISCUSSION

One of the most difficult problems in the study of structural proteins (e.g., silk, collagen, elastin, resilin, and keratin) is solubilization without degradation of the polymer (Lucas et al. 1958). *N. clavipes* dragline silk, like other insect and arachnid fibroins, does not dissolve in water; nor does it solubilize at room temperature in most of the solvents described in Table 1, except for the strong acids and Soluene. Soluene could not conveniently be removed from the silk solution and was deemed unsuitable in any further analysis.

The solubilization effect of hydrochloric/propionic acid treatment on spider silk is almost instantaneous at room temperature. Hydrolysis of the protein backbone does not appear to take place as a result of solubilization in strong acids (6N HCL/Propionic acid). The molecular weight integrity of the polymer was maintained as observed by polyacrylamide gel electrophoresis; a single, homogeneous band of approximately 350,000 daltons was observed, in both acid solubilized reeled silk and from luminal contents isolated from dissected major ampullate glands. Hydrochloric/propionic acid may act as a strong oxidizing agent. The amino acids most affected by oxidation are cysteine, methionine, and tyrosine. Cysteine was initially presumed to be destroyed over time, but the use of hydrolysis controls in the analysis indicated this was not the case. More importantly, it appears that disulfide bridges do not play a role in maintaining

the structural integrity of silk for two reasons: (1) the overall absence of cysteine (<0.50%) in the amino acid analysis, and (2) the insolubility of the silk in mercaptoethanol. Methionine also appears to have little influence on the secondary structure, since the total amount of this amino acid (< 0.50%) is too small and methionine is not implicated in crosslinking in any characterized protein.

The content of tyrosine, however, is more interesting. This amino acid residue appears unaffected in dragline silk hydrolysis and analysis (3.0%). Two plausible hypotheses may be presented, both indicating that tyrosine plays a specific role in preserving the secondary structure of spider silk: (i) spider silk tyrosine is protected against oxidation either by its position inside the hydrophobic moiety of the molecule, or by an electrophilic substitution at the e1 or e2 positions of the phenolic hydroxyl, (ii) any oxidized tyrosines are not completely degraded and complexed in the derivatization reaction, thus remaining unseparated from tyrosine in subsequent analysis. The latter seems unlikely due to the presence of oxygen scavengers in the hydrolysis reaction, which aid in recovery of certain amino acids. The former appears to be logical explanation. Parallel experiments were performed omitting sodium sulfite and hydrolysis controls; subsequently the recovery of tyrosine was unaffected by potential oxidation reactions.

The insolubility of spider silk in 8M urea, 50% lithium bromide, and 1% sodium dodecyl sulfate (Table 1) implies that hydrogen bonding may not be the only mechanism involved in intra-sheet associations between silk molecules, (Seifter and Gallup 1966). This suggests that specific bonding mechanisms which may hold the structure of the fibroin together are unaffected by this treatment. Shaw (1964) and Lucas (1966) have conjectured on the nature of silk intra-sheet bonding, but specific structural and chemical information is still lacking. The absence of cysteine and methionine in the composition of *N. clavipes* dragline silk seems to negate their possible role in the cross-linking of the silk chains. More consistent conclusions are offered by Seifter and Gallup (1966), who state that the structure of silk fibroins may consist of multiple protein regions joined by very specific chemical cross-linkages, although the association between individual silk molecules probably involves both covalent and non-covalent interactions.

The amino acid composition of *N. clavipes* dragline silk depicted in Table 2 shows a uniform trend in chemical composition. In order to determine whether these trends were actually uniform in nature, each spider was silked on three separate occasions as previously described and analyzed in triplicate to yield 9 determinations per spider species. Examination of the data from samples taken from *N. clavipes* show distinct, uniform trends in chemical composition. A wide variation in MaAS amino acid composition was previously reported by Work and Young (1987). It was our conclusion that the lack of variability in the present study was due to the use of extremely sensitive and well defined analytical techniques, high quality instrumentation and the absence of contamination by other silks (e.g., Minor ampullate gland silk). It was therefore concluded that the data illustrates substantial continuity in the chemical composition of major ampullate gland silk from *N. clavipes*.

Table 5 shows the differences in amino acid composition between *B. mori* silk fibroin (cocoon) and *N. clavipes* silk fibroin (MaAS). It can be observed that the composition of the two types of silks differ not only in relative percentages of individual residues, but also in residues present/absent. Two features of the

Table 5.—Comparative data on *Bombyx mori* and *Nephila clavipes* silk fibroins. Data on *B. mori* from Lucas et al. (1955).

Amino acid	<i>Bombyx mori</i>	<i>Nephila clavipes</i> (reeled)
Gly	44.1	37.1
Ala	29.7	21.2
Ser	12.4	4.5
Tyr, Phe	7.5	10.2
Leu, Ile, Val, Asx, Glx	3.6	11.7
Thr	1.2	1.7
Arg	1.5	7.6
Trp	0.5	N/A
Pro	ND	4.5
His, Cys, Lys	ND	1.0
TOTAL	100.0	100.0
Res, short chain (SC)	86.2	62.2
Res, long chain (LC)	13.8	29.8
Ratio (LC/SC)	0.16	0.48

analysis are worth noting; (1) the high percentage of short-chain residues in *Bombyx* fibroin (86.2%) versus *Nephila* fibroin (62.2%), and (2) the 3-fold increase in ratio of LC/SC residues in *Nephila* fibroin (0.16 vs 0.48). These findings may be critical in determining the relative ratios of crystalline-to-amorphous regions in silk, although more empirical evidence is required.

It is routinely believed that in the fibroin of the silkworm *B. mori* there is a consensus sequence of (Gly-X-Gly-X-Gly-X)<sub>n</sub>, where X is alanine or serine, although researchers have generally differed upon the exact amino acid composition of *Bombyx* silk (Lucas et al. 1960; Iizuka 1970; Komatsu 1979; Nadiger et al. 1985). Dickerson and Geis (1969) postulated that the glycine side chains (—H) align themselves opposite alanine (—C<sup>β</sup>H<sub>3</sub>) or serine (—C<sup>β</sup>H<sub>2</sub>O<sub>6</sub>H) side chains to conform to the anti-parallel β-pleated sheet structural model of Pauling and Corey (1953). It should be understood that this applies to the crystalline region of *Bombyx* silk as determined by x-ray diffraction patterns (Iizuka 1965). The high proportion of short side chain amino acids (62%) in the MaAS make it more conceivable for the fiber to attain the conformational structure of the anti-parallel β-pleated sheet. This predicted condition is purely theoretical because the ratios of crystalline-to-amorphous regions in both *B. mori* cocoon silk and *N. clavipes* dragline silk are currently unknown. One can assume that the relative amounts of crystalline and amorphous regions may be determined relative to their physio/chemical properties and their effect on the protein fiber. These assumptions are substantiated by the early work on fibers by Lucas et al. (1955). Interestingly enough we may equate conclusions about physical properties in which small differences induced in the chemical composition of synthetic man-made fibers (e.g., Nylon, Kevlar) translate into significant changes in the physio/chemical properties of the fiber.

The results depicted in table 4 show uniform trends, but clear differences are observed under closer scrutiny. Closer similarities are seen between *Nephila* and *Argiope* than between *Argiope* and *Neoscona* which are from the same family. Although these differences may be ecologically and/or phylogenetically-based. Further analyses of additional species is needed.



The identification of silk gene-related DNA sequences in recombinant organisms may aid in the understanding of the interaction between chemical composition/protein sequence and the exceptional physical properties conferred upon the protein fiber. Studies at the genetic, DNA/protein sequence, and transcriptional/translational control levels will further the understanding of the structure/function relationships of naturally occurring fibers.

### ACKNOWLEDGMENTS

The technical guidance of Ted Tanhauser and Bod Sherwood, Cornell University for solubilization, hydrolysis, and analysis is appreciated. The authors also express their appreciation to Mark Stowe and Angela Choate, University of Florida, Gainesville, FLA for supplying unlimited quantities of spiders. We thank Scott Stockwell who provided helpful comments and suggestions on early drafts of this manuscript.

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*Manuscript received January 1990, revised April 1990.*